Patterns of Genetic Diversity in Remaining Giant Panda Populations

ZHI LU,*† WARREN E. JOHNSON,* MARILYN MENOTTI-RAYMOND,* NAOYA YUHKI,* JANICE S. MARTENSON,* SUSAN MAINKA,‡ HUANG SHI-QIANG,§ ZHENG ZHIHE,** GUANGHAN LI,** WENSHI PAN,† XIARONG MAO,† AND STEPHEN J. O'BRIEN*††

*Laboratory of Genomic Diversity, National Cancer Institute, Frederick, MD 21702-1201, U.S.A.
†Wildlife Research Center, Peking University, Beijing 100671, China
‡Species Survival Commission, Rue Mauverney 28 CH-1196, Gland, Switzerland
§137 Xizhimenwai Street, Beijing Zoo, Beijing, 100044
**Chengdu Research Base of Giant Panda Breeding, Fu Tou Shan, Northern Suburb, Sichuan, China

Abstract: *The giant panda (*Ailuropoda melanoleuca*) is among the more familiar symbols of species conservation. The protection of giant panda populations has been aided recently by the establishment of more and better-managed reserves in existing panda habitat located in six mountain ranges in western China. These remaining populations are becoming increasingly isolated from one another, however, leading to the concern that historic patterns of gene flow will be disrupted and that reduced population sizes will lead to diminished genetic variability. We analyzed four categories of molecular genetic markers (mtDNA restriction-fragment-length polymorphisms [RFLP], mtDNA control region sequences, nuclear multilocus DNA fingerprints, and microsatellite size variation) in giant pandas from three mountain populations (Qionglai, Minshan, and Qinling) to assess current levels of genetic diversity and to detect evidence of historic population subdivisions. The three populations had moderate levels of genetic diversity compared with similarly studied carnivores for all four gene measures, with a slight but consistent reduction in variability apparent in the smaller Qinling population. That population also showed significant differentiation consistent with its isolation since historic times. From a strictly genetic perspective, the giant panda species and the three populations look promising insofar as they have retained a large amount of genetic diversity in each population, although evidence of recent population reduction—likely from habitat loss—is apparent. Ecological management to increase habitat, population expansion, and gene flow would seem an effective strategy to stabilize the decline of this endangered species.*

Patrones de Diversidad Genética en Poblaciones Remanentes de Panda Gigante

Resumen: *El panda gigante (*Ailuropoda melanoleuca*) es uno de los símbolos más familiares de la conservación de especies. La protección de poblaciones de panda gigante ha sido asistida recientemente con el establecimiento de más reservas mejor administradas en los hábitats existentes en seis cadenas montañosas en el occidente de China. Sin embargo, estas poblaciones restantes se están aislando cada vez más, con ello crece la preocupación de que el flujo histórico de genes se alterará y que los reducidos tamaños poblacionales conducirán a una variabilidad genética disminuida. Analizamos cuatro categorías de marcadores genéticos moleculares (polimorfismos de restricción de longitud de fragmentos (PRLF) de mtDNA, secuencias de control de regiones de mtDNA, huellas de DNA nuclear multilocus y variación del tamaño de microsatélites) en pandas gigantes de poblaciones en tres cadenas montañosas (Qionglai, Minshan y Qinling) para evaluar los niveles de diversidad genética y para detectar la evidencia de subdivisiones de poblaciones históricas. Las tres poblaciones tuvieron niveles moderados de diversidad genética comparada con la de carnívoros estudiados similarmente para las cuatro medidas genéticas, con una ligera pero consistente reducción de variabilidad*

††*Address correspondence to S. J. O'Brien, email obrien@ncifcrf.gov*
Paper submitted March 9, 2000; revised manuscript accepted January 31, 2001.

aparente en la población más pequeña de Qinling. Esa población también mostró diferenciación significativa consistente con su aislamiento desde tiempos históricos. Desde una perspectiva estrictamente genética, la especie de panda gigante y las tres poblaciones parecen seguras hasta ahora ya que han retenido una gran diversidad genética en cada población, aunque la evidencia de la reducción poblacional reciente, por pérdida de hábitat, es notable. Así, el manejo ecológico para incrementar el hábitat, la expansión de la población y el flujo de genes parecería una estrategia efectiva para estabilizar la declinación de esta especie en peligro de extinción.

Introduction

Giant pandas (*Ailuropoda melanoleuca*) are indigenous to the People's Republic of China, where they are found in regions of alpine forest on the edge of the Tibetan plateau. Populations originally extended throughout most of southern and eastern China, northern Myanmar, and northern Vietnam, but habitat loss from deforestation during the eighteenth century rapidly reduced their numbers and distribution. Currently, giant pandas are restricted to six forest fragments in the rugged mountain ranges of the eastern edge of the Tibetan plateau in the western Chinese provinces of Gansu, Shaanxi, and Sichuan (Fig. 1) (Schaller et al. 1985; MacKinnon et al. 1989; O'Brien et al. 1994). Within these areas they survive only where there is bamboo, and thus are generally restricted to 1200–3400 m in elevation in what is termed the southwestern China temperate-forest ecoregion. Two surveys conducted in the middle 1970s and 1980s suggested that at least 1100 giant pandas existed in the wild at the time (Schaller et al. 1985; Johnson et al. 1988; MacKinnon et al. 1989). One important conservation measure the Chinese government has taken, with support from international communities, is to expand the protected-area system. The number of giant panda reserves has been increased from 13 in the late 1980s to 33 in the late 1990s. Currently, over 60% of the giant panda's habitat and populations are under protection (MacKinnon et al. 1989).

In spite of their status as a Chinese national treasure and international symbol of the conservation movement, relatively little is known about remaining panda populations. Long-term fieldwork started in 1980 in the Wolong and Tangjiahe Reserves in Sichuan Province (Schaller et al. 1985, 1989; Schaller 1986, 1993) and then in the Qinling Mountains in Shaanxi Province in the mid-1980s (Pan et al. 1988; Lu 1991, 1993; Lu et al. 1994; Pan 1995; Zhu 1996; Zhu 1999; Pan & Lu 2001) provide important insights into the life history, population dynamics, social behavior, and habitat requirements of pandas. This research depicted populations that were increasingly threatened by timber harvest, poaching, and other human pressures, resulting in gradual but deliberate habitat reduction and isolation. Giant panda ranges have been halved in the last 25 years, and the majority of surviving wild populations, estimated at about 25, have fewer than 20 individuals (Johnson et al. 1988; Schaller 1993; MacKinnon et al. 1989). Such small populations are in acute danger of extinction due to chance demographic factors (10–20% of these populations have disappeared since the 1970s) as well as inbreeding and its well-known consequences (O'Brien & Knight 1987; O'Brien 1994*a*, 1994*b*; Frankham 1995; Saccheri et al. 1998; Soulé & Mills 1998). A preliminary report (Su et al. 1993) suggests that giant pandas may have reduced allozyme variation relative to certain bear populations.

We employed four distinct molecular genetic techniques, two that assay genetic variation in the mitochondrial genome and two that measure variation in the nuclear genome, to characterize the extent and differentiation of genetic diversity among individuals from three isolated giant panda populations (Fig. 1). Qinling, the smallest area (1135 km^2) had an estimated 109–240 pandas in 1980s (Pan et al. 1988), Minshan (6127 km^2) had 581 animals, and Qionglai (3355 km^2) had 233 animals (MacKinnon et al. 1989).

The results of our analyses are relevant to the development of objective management plans for both captive and wild panda populations (MacKinnon et al. 1989; Lu et al., 2000). Because of the visibility and symbolic value of pandas in China and abroad, their management has been the subject of great interest and notoriety (Schaller et al. 1985; O'Brien & Knight, 1987; Schaller 1993; O'Brien et al. 1994) and will benefit greatly from a thorough analysis of the genetic structure of remaining populations.

Methods

Samples

Samples were collected from wild animals and from unrelated wild-born, captive animals of known geographic origin (Table 1; Fig. 1). Most samples came from individuals from three main populations from three mountain ranges, Qinling (n = 14), Minshan (n = 7), and Qionglai (n = 15). Two additional samples collected from the Xiangling and Liangshan Mountains were considered part of the Qionglai population for analyses. The Qinling animals derive from a study group from two adjacent valleys that may be related genetically (Lu 1991). Genomic DNA was extracted from frozen leukocytes, primary fi-

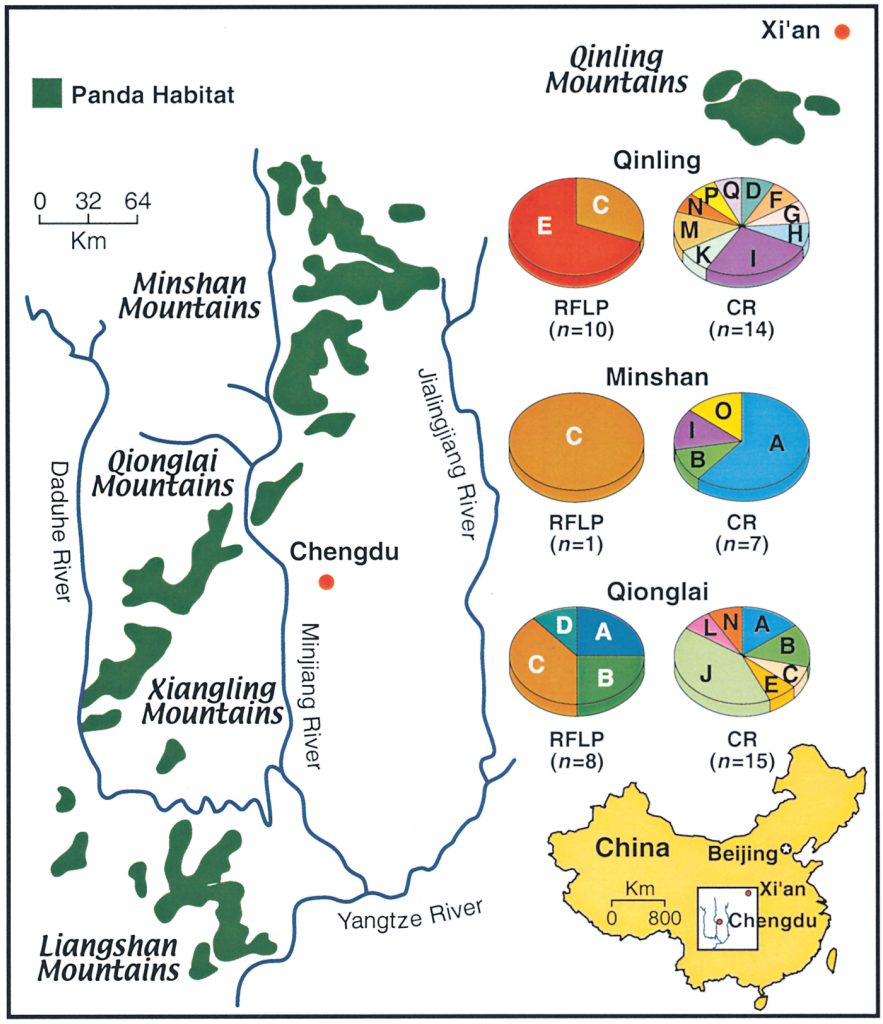

Figure 1. The geographic relationship among giant panda populations sampled for this study. The frequency distribution of mitochrondrial DNA restriction-fragment-length polymorphisms (RFLP) and control-region (CR) haplotypes, defined in Tables 3 and 4 respectively, are specified by letters in pie charts.

broblast cultures from skin biopsies (Sambrook et al. 1989), hair samples (Higuchi et al. 1988), or sperm (Camper et al. 1984).

Mitochondrial DNA variation

Mitochondrial DNA variation was assayed with two techniques. First, we measured mtDNA restriction-fragment-length-polymorphism (RFLP) variation in 19 animals from the three populations (Qionglai, Minshan, Qinling). Genomic DNA (1 μg) from each animal was digested separately with 28 restriction enzymes (monomorphic restriction enzymes: ACCI, APAI, AVAI, AVAII, BAMHI, BCLI, BSTEI, BSTUI, MLUI, DRAI, ECORI, ECORV, HINDIII, KPNI, NCOI, NDEI, PSTI, SMAI, SSTI, SSTII, STUI, XBAI, XHOI; Polymorphic restriction enzymes: CLAI, PVUII, HPAI, STYI, HINCII), separated by electrophoresis in 1% agarose gels, and transferred to nylon filters (UV Duralon; Stratagene) by Southern blotting. The DNA fragments on the membrane were hybridized with a [^{32}P] dCTP–labeled molecular clone of feline mtDNA (Lopez et al. 1994). The mtDNA variation was described by estimating pi, d_{xy}, and d_a with the computer program MAXLINK (Nei & Tajima 1983). Nucleotide diversity, pi, measures the probability that two randomly selected sequences from two individuals within a population will have different nucleotides at a given position (Nei & Li 1979; Nei 1987). The average nucleotide diversity between populations, d_{xy}, is the probability that two randomly selected sequences from two populations will not share the same sites (Nei & Li 1979). The net nucleotide diversity between two populations, d_a, discounts the intrapopulation variation and was calculated as: $d_a = d_{xy} - (p_x + p_y)/2$.

We also measured mtDNA variation by sequencing a 268-bp fragment of the control region with 36 pandas from three populations. Nucleotide sequences were obtained by polymerase chain reaction (PCR) amplification through control region universal primers (Kocher et al. 1989). Thirty cycles of PCR were performed in a programmable heat block (ABI-Perkin-Elmer 9700 Thermal Cycler). Each cycle had 1-minute denaturation at 92° C, 1-minute annealing at 48° C, and 1-minute extension at 72° C. Reactions (100 μL) were prepared with 10 ng genomic DNA in 10 mM Tris-HCl of pH 8.3, 1.5 mM

Table 1. The Ame reference number, sex, name, studbook number, geographic origin, source of animal at time of sample collection, and summary of mtDNA restriction-fragment-length-polymorphism variation (RFLP) and control-region (CR) haplotype, DNA fingerprint, and number of microsatellites genotyped for the giant pandas analyzed in this study.[a]

							mtDNA haplotypes		*Microsatellite*	
Population[b]	*Ame no.*	*Sex*	*Name*	*Stud no.*	*Original location (mt. range)*[b]	*Source*	*RFLP*	*CR*	*DNA-FP*	*no. loci*
Captive										
Minshan										
Min	5	F	Ming Ming	214	Pingwu (min)	London Zoo		A		17
Min	12	F	Nan Nan	271	Nanping (min)	Wolong	C	B		18
Min	48	F	Tang Tang	309	Tangjiahe (min)	Wolong		A		18
Min	49	M	Zhen Zhen	306	Nanping (min)	Wolong		A		18
Min	50	F	Kang Kang	352	Pingwu (min)	Wolong		I		18
Min	74	M	Lin Nan	298	Baishuijiang (min)	Chengdu Zoo		A		18
Min	78	F	Nan Nan	180	Nanping (min)	Chongqing Zoo		O		18
Qionglai										
Qio	1	F	Ling Ling	112	Baoxing (qio)	National Zoological Park, U.S.A.	A	L	X	16
Qio	2	M	Hsing Hsing	121	Baoxing (qio)	National Zoological Park, U.S.A.	B	J	X	16
Qio	4	M	Chia Chia	141	Baoxing (qio)	Mexico Zoo	C	E	X	18
Qio	11	M	Shan Shan	221	Sanjinag (qio)	Wolong	C	B	X	18
Qio	13	M	Le Le	305	Baoxing (qio)	Wolong	B	J	X	18
Qio	14	F	Ching Ching	222	Baoxing (qio)	Wolong	C	B	X	18
Qio	15	?			Qionglai	Mexico Zoo	D			
Xia	18	M	Pe Pe	167	Yuexi (xia)	Mexico Zoo	A	N		16
Qio	41	F	Chun Chun	380	Wolong (qio)	Wolong		C		
Qio	42	M	Zheng He	376	Wenchuan (qio)	Wolong		A		16
Qio	43	M	Shi Shi	381	Wenchuan (qio)	Wolong		J		18
Qio	44	M	Xin Xing	329	Baoxing (qio)	Wolong		J		18
Qio	45	M	Pan Pan	308	Baoxing (qio)	Wolong		J		18
Xia	53	F	Jia Si	365	Ebian (lia)	Wolong		N		18
Qio	75	M	Chuan Chuan	202	Baoxing (qio)	Chongqing Zoo		J		18
Qio	81	M	Xiaopingping	342	Lushan (qio)	Chengdu Zoo		A		18
Qio	92	M	Bao Bao	150	Baoxing (qio)	Beijing Zoo				7
Wild living										
Qinling										
Qin	22	M	1290		Huayang (qin)	Qingling	E	M	X	18
Qin	24	F	Jiao Jiao		Huayang (qin)	Qingling	E	F		18
Qin	25	M	Dabai		Huayang (qin)	Qingling	E	H	X	18
Qin	26	F	Ruixue		Huayang (qin)	Qingling	E	M	X	18
Qin	27	F	Mo Mo		Huayang (qin)	Qingling	C	I	X	18
Qin	28	M	Professor		Huayang (qin)	Qingling	C	I	X	18
Qin	29	M	Oldman-shui		Huayang (qin)	Qingling	E	G	X	18
Qin	30	M	94		Huayang (qin)	Qingling	E	H	X	18
Qin	31	M	Dahuo		Huayang (qin)	Qingling	E	K	X	18
Qin	32	M	Xiaohuo		Huayang (qin)	Qingling		Q	X	18
Qin	33	M	Xinxing		Huayang (qin)	Qingling		N	X	18
Qin	34	F	Nuxia		Huayang (qin)	Qingling	C	I	X	18
Qin	36	F	Fang Fang		Foping (qin)	Qingling		P	X	18
Qin	39	M	Dashun		Huayang (qin)	Qingling		D		18

[a] *All animals except qio 15 were wild-born. Qinling animals were free ranging; the rest were captive individuals from zoos or Wolong Breeding Center.*
[b] *Abbreviations: min, Minshan; qio, Qionglai; qin, Qinling; xia, Xiangling; lia, Liangshan.*

$MgCl_2$, 200 μM dNTP, 2.5 units *Thermus aquaticus* DNA polymerase. Reaction products were resuspended in 2 mL of dH_2O and concentrated with Centricon 100 microconcentrators. Products were sequenced in both forward and reverse directions with a Dye Terminator Prism sequencing kit and an ABI 373A automated DNA sequencer (Applied Biosystems, Inc.), and consensus sequences of forward and reverse sequences were determined. Alignments of the sequences were obtained by the algorithm of Needleman and Wunsch (1970) with the PILEUP program of the Genetics Computer Group computer package version 8 and were visually confirmed. Sequences were deposited in GenBank (accession numbers AF 363507–363524).

Phylogenetic trees were constructed from the sequence data through three methods as implemented in the PAUP* computer program (Swofford 1999). The minimum-evolution tree was estimated by the neighbor-joining (NJ) algorithm (Saitou & Nei 1987) through a distance matrix of Kimura's two-parameter distances (Kimura 1980) and a general heuristic search with tree-bisection-reconnection branch swapping. The maximum parsimony (MP) tree was estimated with a general heuristic search through simple-sequence addition of sequences and tree-bisection-reconnection branch swapping. The maximum-likelihood (ML) tree was generated according to the HKY or F84 model with a starting tree from the ME analysis, empirically derived nucleotide frequencies, and default values for transition/transversion ratios and the shape parameter of the gamma distribution of among-site rate heterogeneity (Nei et al. 1996). Successive ML trees incorporating new estimates of these parameters were obtained iteratively until an optimal tree was derived consistently. Bootstrap resampling (100 iterations) was done with the minimum evolution and maximum parsimony analyses to test the reliability of the data to derive consistent topologies. Minimum Spanning Networks (Excoffier & Smouse 1994) were constructed with Arlequin version 2.000 (Schneider et al. 2000) to depict phylogenetic, geographic, and potential ancestor-descendant relationships among sequences.

Nuclear Genetic Variation

Multilocus DNA fingerprinting was used on a subset of giant pandas to describe diversity in the nuclear genome. The DNA (6 μg) of 6 giant pandas from Qionglai and 12 from Qinling was digested with HINFI and HAEIII, separated by electrophoresis in 1% agarose gels, transferred to nylon filters, and hybridized to the [^{32}P] dCTP–labeled feline-specific minisatellite probes FCZ8 and FCZ9 (Gilbert et al. 1990, 1991). Average number of bands, number of monomorphic loci, heterozygosity (H_e), average percentage difference (APD), and mean average percent difference (MAPD) were estimated as by Stephens et al. (1992).

Eighteen microsatellite loci were identified from a small insert library of giant panda genomic DNA, which we screened with radiolabeled oligonucleotide probes using wash conditions for hybridization filters and sequencing of recombinant clones as described by Dietrich et al. (1992) and Menotti-Raymond et al. (1997). Primer pairs were designed in unique sequence flanking the microsatellite for a T_m of 60° C through the program PRIMER (version 0.5; Lincoln, Daly, Lander, and Whitehead Institute for Biomedical Research, Cambridge, Massachusetts) (Table 2). The PCR amplifications of individual microsatellite loci were performed in 10-μL reaction volumes containing 1× Perkin Elmer PCR buffer (10 mM Tris-hydrochloric acid of pH 8.3, 50 mM potassium chloride), 2 mM $MgCl_2$, 250 μM each of the four deoxyribonucleoside 5′-triphosphates (dATP, dCTP, dGTP, dTTP) (Pharmacia), 0.4 unit AmpliTaq or AmpliTaq Gold DNA polymerase (Perkin-Elmer Cetus), 4.0 picomoles each of forward and reverse primer (Life Technologies and PE Applied Biosystems, Inc.), bovine serum albumin at 0.16 mg/mL final concentration (Sigma A-3294) and 50 ng of DNA. The AmpliTaq Gold DNA polymerase was used in the amplification of Ame-μ21, Ame-μ24, Ame-μ27, and Ame-μ28A. One member of each primer pair was labeled with a fluorescent dye, phosphoramidite. Of 18 microsatellite loci utilized in the study (Table 2), one had a compound repeat motif (Ame-μ22), three had a complex repeat motif (Ame-μ26, Ame-μ28B, and Ame-μ70), and all were CA dinucleotide repeats except Ame-μ70, which had a GATA tetranucleotide repeat. The Ame-μ28A and 28B were isolated together and are separated by several hundred bp.

The PCR amplification was performed in a Perkin Elmer Model 9600 Thermocycler for 1 cycle of 3 minutes at 93° C; 10 cycles at 94° C for 15 seconds, 55° C for 1 second, 72° C for 30 seconds; 20 cycles at 89° C for 15 seconds, 55° C for 15 seconds, 72° C for 30 seconds; and 1 cycle of 72° C for 10 minutes. The PCR products were diluted 1:10 with sterile deionized water, and 2 μL of diluted product were mixed with 4 μL of a gel-loading buffer/standard mixture composed of 6:1:1 ratio of formamide (Sigma), ABI PRISM GeneScan-350 Tamra internal lane standard, and ABI GeneScan loading buffer, respectively. Samples were denatured 3 minutes at 94° C and placed on ice. Two microliters of sample were loaded per lane and electrophoresed in 6% denaturing polyacrylamide gels in an ABI Model 373A Automated DNA Sequencer Apparatus for 3.5 hours at 2500 V, 40mA, and 25W. Allele sizes were estimated with ABI GeneScan (version 1.2.2–1) and Genotyper (version 1.1) software packages, and the Local Southern method (Elder & Southern 1987) was used to generate a best-fit curve from the size standards electrophoresed in each lane.

For each of the three main populations and each microsatellite locus, the homogeneity of allele distribution, occurrence of private or unique alleles, and estimated observed and expected heterozygosity were determined with the computer program MICROSAT (Minch et al. 1997). Departure from Hardy-Weinberg expectations

Table 2. Molecular characterization, primer sequences for PCR amplification, and expected product size of panda microsatellite loci.

Name	*Repeat motif*[a]	*Primer sequence*[b]	*Product sizes*	*Heterozygosity*
Ame-μ5	$(CA)_{15}$	CCCCGAGTTGCTGAGTTTTA † TTTCTTCCTGCTCACACAAGG	131–157	0.42
Ame-μ10	$(CA)_{16}$	ACCGTGCTCTTAATCCCCTT † CCCATGCTTATGAGAAACAGG	138–160	0.61
Ame-μ11	$(CA)_{12}$	† TATGCCACCTGCCCAGAC GATGGAAAGAGTAGAGCCAAGG	228–236	0.44
Ame-μ13	$(CA)_{18}$	GGAAGCATTAAGGAAAACATGC † AATGATGACCATTTCAAACGC	142–171	0.51
Ame-μ14	$(CA)_{17}$	CCACCCAGGCACATCTATCT † TTTACTGTGGTGGAAGTTAGGG	139–147	0.54
Ame-μ15	$(CA)_{13}$	† AAGCAGTTGTTTTTGCTTAGTG TGTCAAAGTATTTGCCTCACA	122–130	0.09
Ame-μ16	$(CA)_{20}$	† CCCACTGCGGAAACAATAAT ATCTCATTCTTTTTTGTGGCTG	132–143	0.38
Ame-μ19	$(CA)_{18}$	† CAGGCAGCACAGCTATACCA CCACCTGATACCTATGCACAT	154–162	0.33
Ame-μ21	$(CA)_{18}$	† TATGAAAAGAGCCCAAATGTCA TAGCTCCATCCACGTTGTTG	156–174	0.66
Ame-μ22	$(CA)_{11}$*	AGGAAACATGTTGCCTTTTCA † AGAGGGCAAATAGGAGGGAA	127–129	0.27
Ame-μ23	$(CA)_{18}$	† TGAGCCAAAAGTAAAAGGCTG TTTGTGGACCTGTTATTCCTTG	138–152	0.56
Ame-μ24	$(CA)_{15}$	ATGCATGACATTTTGGGTAGC † TGAAGACCCTAGATGAAGGCA	248–258	0.35
Ame-μ25	$(CA)_{22}$	CATAATTCCCTGGCAATGCT † TAGCCCGCATTGAAAAATG	219–237	0.49
Ame-μ26	$(CA)_{CMPLX}$**	TTTTCAGGCCTCCGAAAAC † ATTCCCAATAAAGCAAATCAGA	114–120	0.46
Ame-μ27	$(CA)_{12}$	† TTGAAGAAGAAGGAACATTCCC TTTTGCAACTATGTCCCTCAGG	132–150	0.29
Ame-μ28A**	$(CA)_{12}$	CGATTAGTCGTCAGCACTCTG † AAGGGTAACTGCAGGTGGG	118–132	0.32
Ame-μ28B**	$(CA)_{CMPLX}$**	CCAGCATCTGGTCTGAGTGA † CCACCTGCAGTTACCCTTGT	169–183	0.36
Ame-μ70	$(GATA)_{CMPLX}$**	TGATGCCGTAAAAACTGCAA † TTTAACTCTTCTGTAGTATTCC	182–284	0.66

[a] *Compound nucleotide repeat motifs are marked with one asterisk and complex motifs with two asterisks.*
[b] *Labeled primers are marked with a dagger.*

and estimates of population subdivision (F_{ST}) and number of migrants per generation (Nm) were determined with the program ARLEQUIN, version 2.0 (Schneider et al. 2000). Phylogenetic trees were constructed according to the NJ algorithm as implemented in PHYLIP (Version 3.5; Felsenstein 1993), with 100 bootstrap iterations from the microsatellite data from distance matrices estimated through kinship coefficients (Bowcock et al. 1994) and the proportion of shared alleles (Bowcock et al. 1994), as implemented in the computer program MICROSAT (Minch et al. 1997).

Results

Two measures of mtDNA variation were examined in samplings of three panda populations: mtDNA-RFLP and DNA sequence variation of 268 bp from the mtDNA

Table 3. Patterns of mtDNA restriction-fragment-length-site polymorphisms (RFLP) in three giant panda populations.*

RFLP Ame	*CLAI*	*PVUII*	*HPAI*	*STYI*	*HINCII*	*HINCII*	*HINCII*	*HINCII*	*Populations (n)*
A	−	−	+	−	+	+	−	−	Minshan (1), Qionglai (2)
B	−	+	−	+	+	−	−	−	Qionglai (2)
C	+	+	+	+	+	+	−	−	Qionglai (3), Qinling (3)
D	−	+	−	+	−	+	+	−	Qionglai (1)
E	−	+	+	+	−	+	−	+	Qinling (7)

**Plus sign denotes presence and minus sign denotes absence of restriction site.*

control region. The RFLP analysis with 28 enzymes revealed five polymorphic restriction enzymes (CLAI, PVUII, HPAI, STYI, and HINCII) specifying eight variable sites distributed into five haplotypes (Table 3; Fig. 1). Haplotype E was restricted to Qinling; A, B, and D were restricted to Qionglai; and C was found in each population analysis. Qionglai had the largest number of haplotypes at four, compared with two in Qinling. Overall, the estimate of nucleotide diversity (π) for the combined panda populations was 0.22. The phylogenetic relationship was star-like (not shown), and the differences between different haplotypes ranged from three to five mutational steps (Table 3).

Aligned sequences from the control region of 36 pandas (14 from Qinling, 7 from Minshan, and 15 from Qionglai) revealed 17 distinct haplotypes (A–Q) that differed from each other by 1–9 bp (0.4–3.3%) (Table 4). There were 16 variable sites with two insertion/deletions sites. We analyzed the sequences using three phylogenetic approaches (minimum evolution/neighbor joining, maximum parsimony, and maximum likelihood) and a minimum-spanning network (Fig. 2). The phylogenetic signal was not strong, however, and there was no striking correspondence of control-region haplotype with geographic origin. The absence of phylogenetic clustering of both mtDNA–RFLP and control region sequence probably reflects the occurrence of gene flow between the populations until rather recently, because no geographic differentiation is apparent.

Nuclear minisatellite loci revealed a moderate level of variation in a small sampling of giant pandas from the Qionglai (H_e = 35.8%; n = 6) and Qinling (H_e = 34.5; n = 12) populations (Table 5), which is much higher, for example, than comparable estimates based on the same probes from the genetically uniform Gir forest lion (H_e = 2.9%; Gilbert et al. 1991), Florida panther (H_e = 10.5%; Roelke et al. 1993), or island fox (H_e = 0.4%; Gilbert et al. 1990). Giant pandas more closely resemble DNA fingerprint heterozygosities observed in outbred lions (H_e = 49.1%) or African cheetah (*Acinonyx jubatus*) populations (H_e = 43.4%) that have reconstituted DNA–fingerprint variations over a 10,000-year interval since their last extreme population bottleneck (Stephens et al. 1992; Menotti-Raymond & O'Brien 1993). There was a suggestion of higher diversity in Qionglai (H_e = 30.1–41.4%, MAPD = 38.3%) than in Qinling (H_e = 26.3–46.5%, MAPD = 31.5%), paralleling the slight reduction in mtDNA haplotype counts between the two populations (Table 5).

To extend the phylogenetic and quantitative population-diversity assessment, we developed and characterized 18 microsatellite loci specific to the giant panda in the three populations (Table 2). Each population displayed moderate to high microsatellite variation compared with other species. Average heterozygosity ranged from 49% to 58% in the three populations, with a total of 106 alleles observed across the 18 loci (Table 5). There were multiple population-specific signature alleles in each population, which lead to a mean of 1.7–4.0 signature alleles retained in the composite microsatellite genotype of any individual (Table 5). Qinling and Qionglai had nearly twice as many signature alleles as Minshan in each population. The allele-size expansion, termed microsatellite variance (a measure of the breadth of allele-size range for a polymorphic microsatellite locus), was appreciable in each population (Table 5) (Goldstein &

Table 4. Patterns of mtDNA control-region (CR) haplotypes in three giant panda populations.

	Variable nucleotide site																
mtDNA–CR haplotype	*14*	*31*	*34*	*54*	*66*	*111*	*133*	*135*	*152*	*161*	*176*	*228*	*238*	*244*	*245*	*254*	*Population (*n*)*
A	A	T	A	C	C	C	A	C	—	—	C	C	G	T	C	A	Minshan (4), Qionglai(2)
B	A	T	T	C	C	C	A	C	—	—	C	C	G	T	C	A	Minshan (1), Qionglai(2)
C	A	T	A	C	C	C	A	C	—	—	C	C	C	T	C	A	Qionglai(1)
D	A	T	A	C	T	C	A	C	—	—	C	T	G	T	C	A	Qinling (1)
E	A	T	C	T	C	C	A	C	—	—	C	C	G	T	C	T	Qionglai(1)
F	A	T	A	C	T	C	G	C	—	—	C	C	G	C	C	A	Qinling (1)
G	A	T	T	C	T	C	G	C	—	C	C	C	G	T	C	A	Qinling (1)
H	A	T	A	C	T	C	G	C	—	C	C	C	G	T	C	A	Qinling (2)
I	A	T	A	C	C	C	A	C	—	C	C	C	G	T	C	A	Minshan (1), Qinling (3)
J	A	T	A	C	T	T	A	C	—	C	C	C	G	T	C	A	Qionglai(6)
K	A	T	A	C	T	C	A	C	—	C	C	C	G	T	C	A	Qinling (1)
L	C	T	A	C	T	C	A	C	—	C	C	C	G	T	C	A	Qionglai(1)
M	A	T	A	C	T	C	G	G	—	C	G	C	G	T	C	A	Qinling (2)
N	A	T	A	C	T	C	A	C	C	C	C	C	G	T	C	A	Qionglai(2), Qinling (1)
O	A	T	A	C	C	C	A	C	C	C	C	C	G	T	C	A	Minshan (1)
P	A	T	A	C	T	T	A	C	C	C	C	C	G	T	C	A	Qinling (1)
Q	A	C	T	C	T	C	A	C	C	C	C	C	G	C	G	A	Qinling (1)

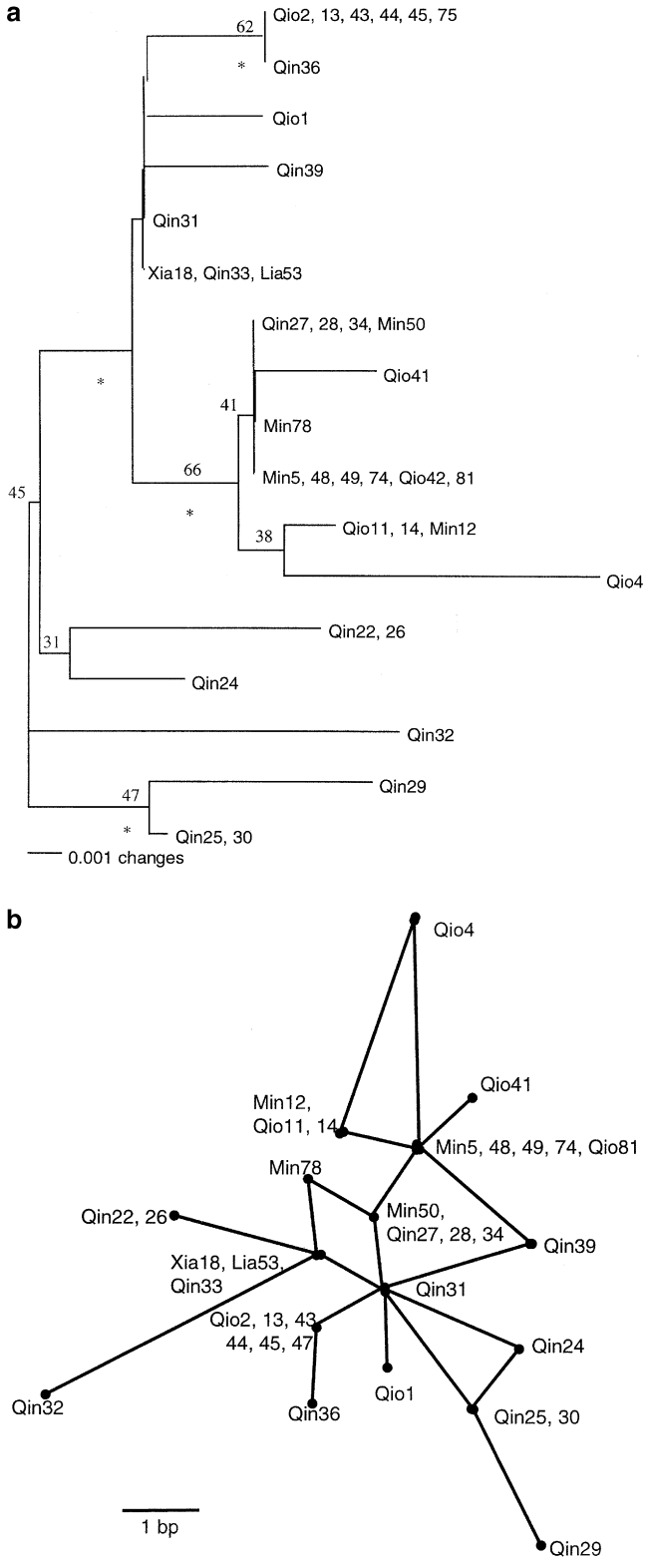

Figure 2. Phylogenetic relationships among mtDNA control-region-sequence haplotypes. (a) Minimum evolution tree constructed by means of a Kimura 2-parameter distance matrix and the neighbor-joining algorithm; one of the six best trees found in a heuristic search. Nodes with bootstrap percentages above 40% are listed. Nodes supported by maximum likelihood analyses are labeled with an asterisk. (b) Minimum-spanning network with length of connecting lines proportional to the number of steps between haplotypes, and with haplotypes (filled circles) indicating the individuals and population bearing each haplotype. Individuals are coded by their affiliation with one of five populations; Min, Minshan; Qin, Qinling; Qio, Qionglai; Xia, Xiangling; Lia, Liangshan.

Pollock 1997). This observation is consistent with the supposition of panmixia in recent times for each population and for pandas as a whole. All microsatellite loci conformed to Hardy-Wienberg equilibrium.

The relationships among individual giant pandas were examined in a phylogenetic analysis of composite microsatellite genotypes of 36 individuals (selected from each population and excluding known relatives). Minimum evolution topologies were estimated by the neighbor-joining algorithm based on two genetic-distance measures, percent allele sharing (Dps) and mean kinship (Dkf), which were previously determined applicable to closely related populations (Goldstein et al. 1995; C. A. Driscoll & S. J. O., unpublished data). These phylogenetic trees recapitulated geographic origins with only a few exceptions (Fig. 3), although the statistical bootstrap support for the major nodes was low (20% between Qinling individuals and the others). The Qinling individuals clustered together without exceptions. The Qionglai and Minshan populations were intermingled and the individual specimens from Lingshan and Xiangling were clustered with the Qionglai individuals.

The apparent population differentiation indicated by the phylogeographic microsatellite analysis (Fig. 3) was also apparent in the F_{ST} estimates, which indicated significant differentiation between Qinling and Qionglai (F_{ST} = 0.18; $p < 0.05$) and between Qinling and Minshan (F_{ST} = 0.18; $p < 0.05$), but not between Minshan and Qionglai (F_{ST} = 0.07; $p > 0.05$). These measures imply that Qinling has experienced moderate geographic isolation, as revealed by the microsatellites but not by the mtDNA result. Gene flow has occurred until recently among the two southern populations (Fig. 1), however, because there was no robust geographic substructure between them by any measure (Figs. 1–3).

Discussion

Results of the analyses of genetic variation in pandas based on four different kinds of molecular genetic markers provided insights into the recent evolutionary history of remaining populations. The mtDNA RFLP variation in giant pandas (π = 0.22) was moderate to low compared with other outbred carnivores. For example, compara-

Table 5. Measures of molecular genetic diversity based on mitochondrial restriction-fragment-length-polymorphism variation (mtRFLP), mtDNA control-region sequences, nuclear DNA minisatellite fingerprint variation, and microsatellite variation at 17 loci for three giant panda populations.

	Population			
*DNA marker**	*Qinling*	*Minshan*	*Qionglai*	*all pandas*
mtDNA RFLP				
sample size	10	1	7	19
haplotypes	2	1	4	5
π	0.12	—	0.24	0.22
mtDNA sequences				
sample size	14	7	15	36
haplotypes	10	4	7	17
π	0.04	0.01	0.03	0.06
DNA fingerprints				
sample size	12	—	6	18
HAEIII/Fcz8				
H_e(%)	39.0	—	41.4	39.8
APD	36.9	—	41.3	38.3
HINfI/Fcz8				
H_e(%)	46.5	—	35.5	42.8
APD	39.3	—	39.0	39.2
HAEIII/Fcz9				
H_e(%)	27.7	—	30.1	28.5
APD	24.7	—	34.7	28.0
HINfI/Fcz9				
H_e(%)	26.3	—	36.3	29.6
APD	25.1	—	38.2	29.4
Microsatellite	31.5	—	38.3	33.7
sample size	14	7	15	36
no. alleles	33	35	38	106
observed heterozygosity (%)	57	58	49	44
variance	8.4	9.3	12.6	10.2
average no. alleles/locus	3.3	3.5	4.3	3.7
signature alleles number	15	7	14	36
mean/individual	4.0	1.7	1.5	2.4
range				

**The* H_e *is the estimated average heterozygosity based on Hardy-Wienberg distribution of allele frequencies, and APD is average percent difference in band sharing (Stephens et al. 1992).*

ble estimates of mtDNA RFLP nucleotide diversity (π) range from 0.18 in cheetahs (*Acinonyx jubatus*) (Menotti-Raymond & O'Brien 1993) and 0.35 in North American pumas (*Puma concolor*) (O'Brien et al. 1990) to 1.30 in leopards (*Panthera pardus*) (Miththapala et al. 1996) and 8.00 in black-back jackals (*Canis mesomelas*) (Wayne et al. 1991). Measures of nuclear minisatellite genetic variation of giant pandas were comparable to those of other outbred carnivores. Estimates of heterozygosity in pandas ranged from 0.277 to 0.465 depending on the enzyme and probe used (Table 5). Cheetah heterozygosity is 0.43 (Menotti-Raymond & O'Brien 1993), Serengeti and Ngorongoro lion heterozygosities are 0.435 and 0.491, respectively (Stephens et al. 1992), and domestic cat heterozygosity is 0.449 (Stephens et al. 1992). Microsatellite average heterozygosity is 0.44 in giant pandas, 0.443 in cheetahs, 0.357 in pumas, and 0.373 in lions (C. A. Driscoll & S. J. O., unpublished data).

There was little pattern in the distribution of genetic variation among the three main populations. The Qionglai population had the most mtDNA–RFLP haplotypes (four) (Qinling had 2) and the highest mean average percent difference in fingerprint variation (MAPD = 38.3%) (MAPD = 31.2% in Qinling). Qionglai had an estimated microsatellite heterozygosity of 0.49, whereas Minshan had 0.58 and Qingling had 0.57.

There was also evidence that gene flow between Qinling and the other two populations has been limited for a sufficient period of time to allow them to accumulate unique characteristics. Qinling was the only population with a population-specific mtDNA–RFLP genotype (E) and had the most unique control-region-sequence haplotypes, eight, compared with four in Qionglai and one in Minshan (Fig. 1). Qinling also had a large number of population-specific microsatellite signature alleles (15) compared with Qionglai (14) and Minshan (7) (Table 5). Population differentiation, a measure of interrupted gene flow, was also apparent in the significant F_{ST} values between Qinling and the other populations.

Together, these data indicate that the Minjiang River (Fig. 1) has not been a barrier to gene flow between the populations of the Minshan mountains and the Qionglai, Xiangling, and Liangshan mountains to the southwest. Our results suggest, however, that the Qinling mountain

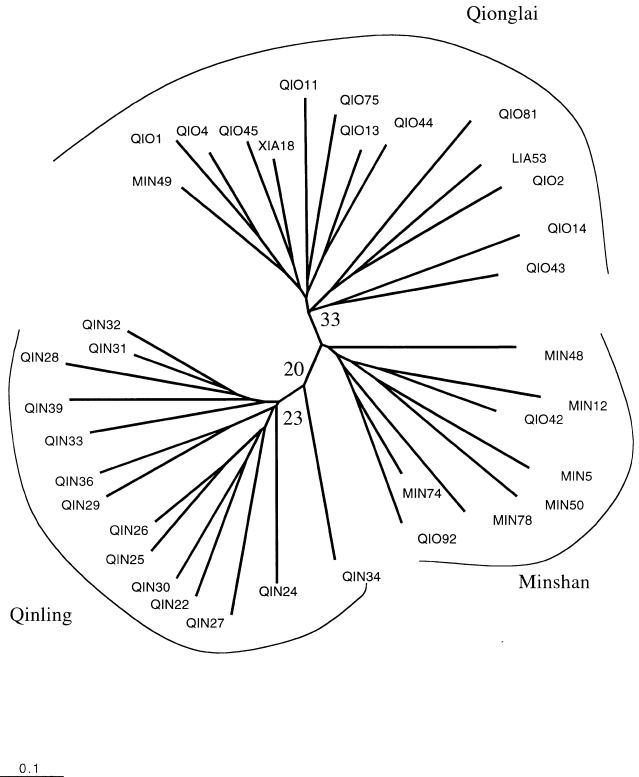

Figure 3. Phylogenetic relationships among 36 giant pandas constructed from 18 microsatellite loci. Unrooted neighbor-joining tree based on mean kinship, Dkf genetic distances with a p-ps transformation. Individuals are coded by their affiliation with one of five populations; MIN, Minshan; QIN, Qinling; QIO, Qionglai; XIA, Xiangling; LIA, Liangshan. Individuals from Xiangling and Liangshan are combined with Qionglai in other analyses.

population became isolated from the other populations in the recent past. This reduction in gene flow did not occur long enough ago for significant differences to become apparent in the mitochondrial markers given historic population sizes. The large number of mtDNA–CR haplotypes in Qinling (Fig. 1) implies that it descends from a large, expanding historic population that with time became isolated and reduced in number and in relative genomic diversity. Such a pattern may signal the beginning of a reversible but damaging trend that can precede genetic and demographic reductions in isolated populations of threatened species.

Management Implications

Our results consistently suggest that the studied panda populations were probably connected until recently, when gene flow between Qinling and the populations of the Minshan and Qionglai mountains was reduced. The date is difficult to estimate precisely, but by comparison to coalescent calculation in other carnivores (Menotti-Raymond & O'Brien 1993; Culver et al. 2000; C. A. Driscoll & S. J. O., unpublished data) it is on the order of a few thousand years. Levels of molecular genetic variation in giant pandas are comparable to those of other carnivores and only slightly reduced in Qinling relative to Minshan and Qionglai, probably reflecting a relatively recent decline in effective population size. Although we find unique mtDNA and microsatellite genotypes in each of the three populations, together the results from these four genetic markers suggest that there is no imperative to manage remaining panda populations as separate units. As much as is practical, gene flow should be encouraged between isolated populations on both sides of the Minjiang River, either through the maintenance or reestablishment of natural corridors or, in demographically critical populations, through intervention.

Contact between pandas in the Qinling mountains and more-southern populations has probably been curtailed effectively. The effect of maintaining current management practices will be to reinforce more recently derived differences between Qinling and other panda populations and not differences in more-historic population patterns. A more important concern, however, may be the maintenance of a long-term viable population in Qinling, given current and projected effective population sizes. A detailed analysis is needed to determine the effect the demographic structure of this population may have on genetic variation.

The genetic data raise some provocative questions about the recent history of the populations. The giant panda's habitat is traversed by several large river systems (Fig. 1; Yangtze, Minjiang, Jialingjiang, and Daduhe), which historically may have posed barriers to migration. But the genetic similarity between the populations implies that gene flow has occurred between the populations until recently, when the valley became settled by Chinese peoples. Together these observations suggest that human activities have posed a more effective migration barrier over the past few thousand years than have the ancient river systems.

Ultimately, these molecular genetic results may offer some comfort to managers of the giant panda. From a genetic perspective, there appear to be no major reductions in the genomic diversity of giant panda populations, although the large number of remaining mtDNA haplotypes in Qinling suggest that this population has likely experienced modest genetic losses from an ancestral, much larger, and genetically diverse population. Nonetheless, long-term field observations of the Qinling population reveal that panda reproduction is not noticeably diminished but is comparable to similarly studied American black bear (*Ursus americanus*) populations (Lu 1991; Zhu 1999; Lu et al. 2000). So far, evidence of inbreeding in Qinling has not been apparent, suggesting

that conservation imperatives should focus on expanding habitat and protecting existing populations from threatened demographic losses due to timber harvest and poaching. Unfortunately, habitat loss and small-population isolation are common across the giant panda's range (MacKinnon et al. 1989; O'Brien et al. 1994), and these acute population reductions need to be identified and reversed.

The molecular genetic data we describe have allowed an interpretation of the consequences of depletion of giant panda habitat and populations in historic times. The same molecular genetic markers may also be applied to parentage assessment of captive and free-ranging populations and to ecological genetic population monitoring based on fecal and hair samples. Each of these applications has the potential to aid managers in designing conservation strategies for this species.

Acknowledgments

We appreciate the assistance of Q. Dagong and M. Xiaorong at Peking University; the staffs of the Beijing Zoo, the Chengdu Zoo, and the Wolong Nature Reserve; and Z. Qingquo and all the individuals listed in Table 1 who aided in the collection of biological samples. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, and the mention of trade names, commercial products, or organizations does not imply endorsement by the U.S. government.

All tissue samples were collected in full compliance with specific U.S. Fish and Wildlife permits (Convention on International Trade in Endangered Species of Wild Fauna and Flora [CITES]; Endangered and Threatened Species) issued to the National Cancer Institute of the National Institutes of Health (principal officer S. J. O'Brien) by the U. S. Fish and Wildlife Service of the Department of the Interior.

Literature Cited

Bowcock, A. M., A. Ruiz-Linares, J. Tomfohrde, E. Minch, J. R. Kidd, and L. L. Cavalli-Sforza. 1994. High resolution human evolutionary trees with polymorphic microsatellites. Nature **368:**455–457.

Camper, S. A., D. N. Luck, Y. Yvonne, R. P. Woychich, T. G. Goodwin, R. H. Lyons Jr., and F. M. Rottmann. 1984. Characterization of the bovine prolactin gene. DNA **3:**237–249.

Culver, M., W. E. Johnson, J. Pecon Slattery, M. Menotti-Raymond, M. Roelke-Parker, and S. J. O'Brien. 2000. Genetic evidence for a South American origin of modern pumas (*Puma concolor*): a phylogeographic study using mitochondrial DNA and microsatellites. Journal of Heredity **91:**186–197.

Dietrich W., H. Katz, S. Lincoln, H. S. Shin, J. Fiedman, N. C. Dracopoli, and E. S. Lander. 1992. A genetic map of the mouse suitable for typing intraspecific crosses. Genetics **131:**423–447.

Elder, J. K., and E. M. Southern. 1987. Computer-aided analysis of one-dimensional restriction fragment gels. Pages 165–172 in M. J. Bishop and C. J. Rawlings, editors. Nucleic acid and protein sequence analysis: a practical approach. IRL Press, Oxford, United Kingdom.

Excoffier, L., and P. Smouse. 1994. Using allele frequencies and geographic divisions to reconstruct gene geneologies within a species: molecular variance parsimony. Genetics **136:**343–359.

Felsenstein, J. 1993. Phylogeny inference package (PHYLIP). Version 3.5. University of Washington, Seattle.

Frankham, R. 1995. Conservation genetics. Annual Review of Genetics **29:**305–328.

Gilbert, D. A., N. Lehman, S. J. O'Brien, and R. K. Wayne. 1990. Genetic fingerprinting reflects population differentiation in the California Channel Island fox. Nature **344:**764–767.

Gilbert, D. A., C. Packer, A. E. Pusey, J. C. Stephens, and S. J. O'Brien. 1991. Analytical DNA fingerprinting in lions: parentage, genetic diversity, and kinship. Journal of Heredity **82:**378–386.

Goldstein, D. B., and D. D. Pollock. 1997. Launching microsatellites: a review of mutation processes and methods of phylogenetic interference. Journal of Heredity **88:** 335–342.

Goldstein, D. B., A. Ruiz-Linares, L. L. Cavalli-Sforza, and M. Feldman. 1995. Genetic absolute dating based on microsatellites and the origin of modern humans. Proceedings of the National Academy of Science of the United States of America **92:**6720–6727.

Higuchi, R., C. H. von Beroldingen, G. F. Sensabaugh, and H. A. Erlich. 1988. DNA typing from single hairs. Nature **332:**543–546.

Johnson, K., G. Schaller, and H. Jinchu. 1988. Responses of giant pandas to a bamboo die-off. National Geographic Research **4:**161–177.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution **16:**111–120.

Kocher, T. D., W. K. Thomas, A. Meyer, S. V. Edwards, S. Paabo, F. X. Villablanca, and A. C. Wilson. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proceedings of National Academy of Sciences of the United States of America **86:**6196–6200.

Lopez, J. V., N. Yuhki, R. Masuda, W. S. Modi, and S. J. O'Brien. 1994. *Numt*, a recent transfer and tandem amplification of mitochondrial DNA to the nuclear genome of the domestic cat. Journal of Molecular Evolution **39:**174–190.

Lu, Z. 1991. The movement pattern, population dynamics and social behaviour of the Giant Panda in Qinling. Ph.D. thesis. Peking University, Beijing. (In Chinese.)

Lu, Z. 1993. Newborn panda in the wild. National Geographic **183:**60–65.

Lu, Z., W. Pan, and J. Harkness. 1994. Mother-cub relationship in the giant panda in Qinling Mountains: comments on rescuing abandoned cubs. Zoo Biology **187:**157–158.

Lu, Z., X. Zhu, D. J. Wang, H. Wang, and W. Pan. 2000. What has the panda taught us? Pages 325–334 in A. Entwistle, editor. Priorities for conservation of mammalian diversity. Cambridge University Press, Cambridge, United Kingdom.

MacKinnon, J., F. Bi, and M. Qiu. 1989. The national conservation management plan for the giant panda and its habitat. World Wildlife Fund and the Ministry of Forestry, Beijing, People's Republic of China.

Menotti-Raymond, M., and S. J. O'Brien. 1993. Dating the genetic bottleneck of the African cheetah. Proceedings of the National Academy of Science of the United States of America **90:**3172–3176.

Menotti-Raymond, M., V. A. David, J. C. Stephens, L. A. Lyons, and S. J. O'Brien. 1997. Genetic individualization of domestic cats using feline STR loci for forensic applications. Journal of Forensic Science **42:**1039–1051.

Minch, E., A. Ruiz-Linares, D. B. Goldstein, M. W. Feldmen, and L. L. Cavalli-Sforza. 1997. MICROSAT. Version 1.5d. Stanford University, Stanford, California. (Available from *http://lotka.stanford.edu/microsat.html.*)

Miththapala, S., J. Seidensticker, and S. J. O'Brien. 1996. Phylogeographic subspecies recognition in leopards (*Panthera pardus*): molecular genetic variation. Conservation Biology **10:**1115–1132.

Nei, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York.

Nei, M., and W. H. Li. 1979. Mathematical model for studying genetic variation in the terms of restriction endonucleases. Proceedings of National Academy of Sciences of the United States of America **76:** 5269–5273.

Nei, M., and F. Tajima. 1983. Maximum likelihood estimation of the number of nucleotide substitutions from restriction sites data. Genetics **105:**207–217.

Nei, M., J. C. Stephens, and N. Saitou. 1996. Methods for computing the standard errors of branching points in an evolutionary tree and their application to molecular data from humans and apes. Molecular Biology and Evolution **2:**66–85.

O'Brien, S. J., and J. A. Knight. 1987. The future of the giant panda. Nature **325:**758–759.

O'Brien, S. J. 1994*a*. A role for molecular genetics in biological conservation. Proceedings of the National Academy of Science of the United States of America **91:**5748–5755.

O'Brien, S. J. 1994*b*. Genetic and phylogenetic analyses of endangered species. Annual Review of Genetics **28:**467–489.

O'Brien, S. J., M. E. Roelke, N. Yuhki, K. W. Richards, W. E. Johnson, W. L. Franklin, A. E. Anderson, O. L. Bass Jr., R. C. Belden, and J. S. Martenson. 1990. Genetic introgression within the Florida panther *Felis concolor coryi.* National. Geographic Research **6:**485–494.

O'Brien, S. J., W. Pan, and Z. Lu. 1994. Pandas, people and policy. Nature **369:**179–180.

Pan, W. 1995. New hope for China's pandas. National Geographic **187:**100–115.

Pan, W., and Z. Lhu. 2001. A chance of lasting survival. Peking University Press, Beijing.

Pan, W., Z. Gao, Z. Lu, et al. 1988. The giant panda's natural refuge in Qinling. Peking University Press, Peking.

Roelke, M. E., J. S. Martenson, and S. J. O'Brien. 1993. The consequences of demographic reduction and genetic depletion in the endangered Florida panther. Current Biology **3:**340–350.

Saccheri, I., M. Kuussaari, M. Kankare, P. Vikman, W. Fortellus, and I. Hanski. 1998. Inbreeding and extinction in a butterfly metapopulation. Nature **392:**491–494.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution **4:**406–426.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a lab manual. 2nd edition. Cold Spring Harbor Laboratory Publications, Cold Spring Harbor, New York.

Schaller, G. 1986. Secrets of the wild panda. National Geographic **169:** 284–309.

Schaller, G. 1993. The last panda. University of Chicago Press, Chicago.

Schaller, G., H. Jinchu, W. Pan, and Z. Jing. 1985. The giant panda of Wolong. University of Chicago Press, Chicago.

Schaller, G., Q. Teng, K. Johnson, X. Wang, H. Shen, and J. Hu. 1989. The feeding ecology of giant panda and Asiatic black bear in the Tangjiahe Reserve, China. Pages 212–241 in J. Gittleman, editor. Carnivore behavior, ecology, evolution. Cornell University Press, Ithaca, New York.

Schneider, S., D. Roessli, and L. Excoffier. 2000. ARLEQUIN. Version 2.000. University of Geneva, Switzerland.

Soulé, M. E, and L. S. Mills. 1998. No need to isolate genetics. Science **282:**1658–1659.

Stephens, J. C., D. A. Gilbert, N. Yuhki, and S. J. O'Brien. 1992. Estimation of heterozygosity for single-probe, multilocus DNA fingerprints. Molecular Biology and Evolution **9:**729–743.

Su, B., L. Shi, G. He, A. Zhang, Y. Song, and L. Fei. 1993. Genetic diversity in the giant panda: evidence from protein electrophoresis. Pages 292–296 in Z. Anju and H. Guangxin, editors. Minutes of an international symposium on the protection of the giant panda. Chengdu Research Base for Giant Panda Breeding, Chengdu, China.

Swofford, D. L. 1999. PAUP phylogenetic analysis using parsimony and other methods. Computer program. Sinauer Associates, Sunderland, Massachusetts.

Wayne, R. K., B. Van Valkenburgh, and S. J. O'Brien. 1991. Molecular distance and divergence time in carnivores and primates. Molecular Biology and Evolution **8:**297–319.

Zhu, X. 1996. Vocalization analysis of the giant panda cubs. M.S. thesis. Peking University, Beijing.

Zhu, X. 1999. Social behavior and reproductive strategy of the giant panda in Qinling. Ph.D. thesis. Peking University, Beijing.